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Characterization of a Macaque Recombinant Monoclonal Antibody That Binds to a CD4-Induced Epitope and Neutralizes Simian Immunodeficiency Virus

JOAKIM GLAMANN AND VANESSA M. HIRSCH*

Laboratory of Molecular Microbiology, National Institute of Allergy and Infectious Diseases, National Institutes of Health, Rockville, Maryland 20852

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A potent neutralizing Fab fragment from a long-term survivor of simian immunodeficiency virus (SIVsm) infection was used to construct a recombinant macaque immunoglobulin G1κ (IgG1κ) molecule, designated IgG1-201. A Chinese hamster ovary cell line expressing IgG1-201 was derived by stable transfection and optimized for antibody secretion by methotrexate selection and dihydrofolate reductase gene amplification. IgG1-201 effectively neutralized the homologous, molecularly cloned SIVsmH4 virus but had no activity against the heterologous SIVmac251/BK28 virus. The previously characterized, neutralization-resistant SIVsmE543-3 virus was also not neutralized by IgG1-201. Binding to SIVsmH4 gp120 was enhanced in the presence of recombinant soluble CD4, suggesting that IgG1-201 bound a CD4-induced epitope. IgG1-201 immunoprecipitated the SIVsmH4 but not the SIVsmE543-3 envelope despite a close relationship between these two clones. Immunoprecipitation of a panel of SIVsmH4/SIVsmE543-3 chimeric viruses tentatively assigned the neutralization epitope to the third constant domain, immediately C terminal to the V3 loop. These findings suggest the presence of at least one CD4-induced neutralization epitope on SIV, as is the case with human immunodeficiency virus type 1.

Initial efforts to develop an AIDS vaccine focused upon eliciting neutralizing antibodies using envelope-based immunogens. More recent efforts have included additional viral gene products with the goal of generating both cytotoxic T lymphocytes and neutralizing antibodies (5, 27, 33). The ability to generate a neutralizing antibody response that is capable of broadly neutralizing primary isolates of human immunodeficiency virus (HIV) has proven difficult and remains a major goal (5, 33). Despite the difficulties in generating a broadly neutralizing antibody response through immunization, passive immunoprophylaxis experiments with a number of different animal models have provided evidence that, under certain circumstances, neutralizing antibodies are indeed capable of preventing or modulating infection. A number of human monoclonal antibodies have been generated that, alone or in combination, effectively neutralize primary HIV type 1 (HIV-1) isolates in vitro (4, 10, 12, 23, 30, 31, 34, 40, 41). Some of these HIV-1 human monoclonal antibodies can protect against infection in the hu-PBL SCID model (1, 13, 35, 36). Passive infusion of a human monoclonal antibody specific for a conserved epitope on gp41 did not prevent infection of chimpanzees, but the treated animals controlled viremia better (9). Most convincingly, recent studies have demonstrated that neutralizing antibodies provided passively by human monoclonal antibodies alone or in combination with HIV immunoglobulin (Ig) can be highly effective in blocking infection in a pathogenic simian/human immunodeficiency virus (SHIV) macaque model (32). In a similar vein, IgG purified from an HIV-1-infected chimpanzee was effective in blocking SHIV infection of macaques (39).

Fewer studies have been performed with the simian immunodeficiency virus (SIV) macaque model (8, 15, 22, 29, 37, 43) due to a paucity in macaque monoclonal antibodies (14, 38). However, many of the neutralizing epitopes for SIV have been partially mapped by peptide scanning (20, 21) and mutational analysis (3, 6, 7, 25, 44). A number of studies conducted with variants of the SIVmac lineage have shown that discrete amino acid substitutions in the envelope glycoprotein can alter the neutralization phenotype (3, 25, 44). Such changes include the acquisition of novel glycosylation sites as a viral mechanism for avoiding recognition by neutralizing antibody (6).

In a previous report, we described the isolation of Fab fragments from a long-term survivor of SIVsm infection using phage display technology (2, 14). One of these Fab fragments, Fab 201, potently neutralized homologous SIVsm isolates but was ineffective in neutralizing the heterologous SIVmac isolates (14). Fab 201 competed with the mouse monoclonal antibodies, KK5 and KK9, that react with a conformational-dependent epitope on the V3 to V4 region of the SIV envelope (20, 21). In an effort to develop a SIV-specific antibody reagent that is suitable for passive immunotherapy trials, the present report describes the conversion of Fab 201 into a recombinant macaque IgG1κ molecule and its unique biological properties. In order to generate a neutralizing macaque monoclonal IgG antibody, it was necessary to (i) generate macaque heavy chain immunoglobulin genes, (ii) insert macaque heavy and light chain genes into appropriate eukaryotic expression vectors, and (iii) develop stable transfectants that expressed the recombinant IgG.

Rhesus macaque heavy chain immunoglobulin genes were amplified by PCR from bone marrow cDNA of RhE544, the macaque that was the source of Fab 201 (14). The PCR amplification used two N-terminal primers (5′-AGG TGC AGC TGC TCG AGT CTG G-3′ and 5′-CAG GTG CAG CTG CTC GAG TCG GG-3′) (13) and a single primer corresponding to the carboxy-terminal end of the fourth constant domain (5′-ATC ATA CGT AGA TAT CTA TCA TTT ACC CGG

* Corresponding author. Mailing address: NIAID Twinbrook II Facility, 12441 Parklawn Dr., Rockville, MD 20852. Phone: (301) 496-2976. Fax: (301) 480-2618. E-mail: vhirsch@niaid.nih.gov.

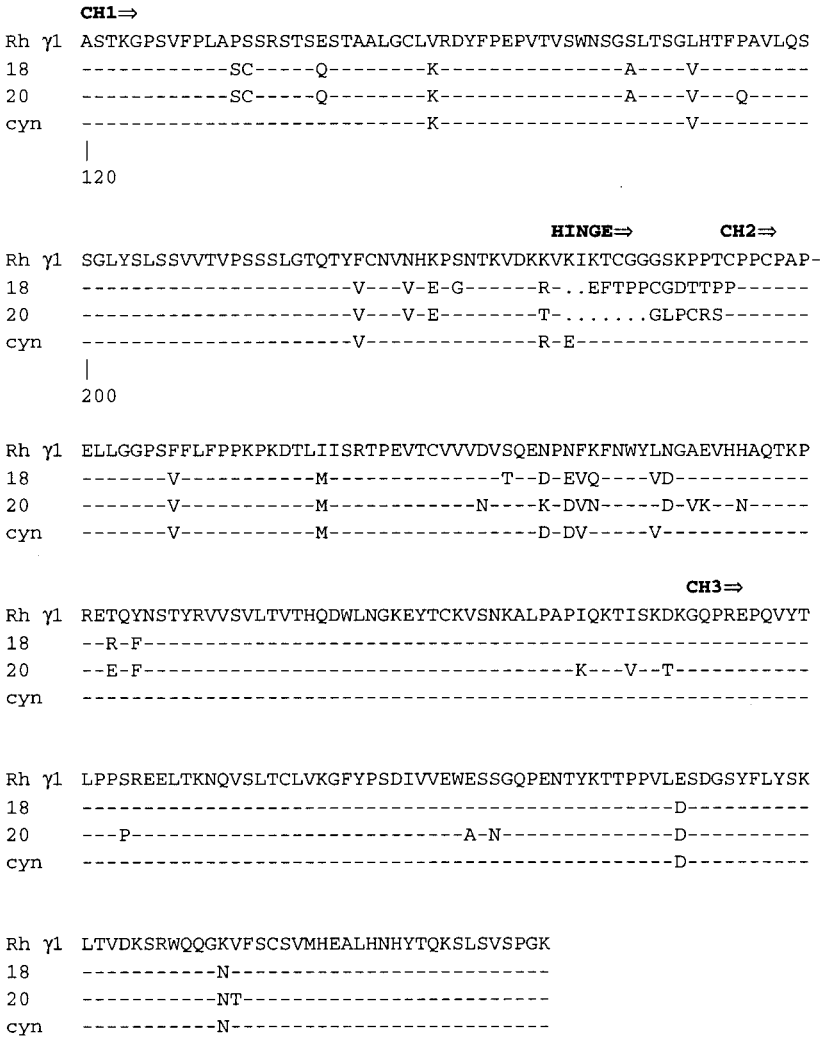

FIG. 1. Alignment of rhesus monkey C_H region amino acid sequences with the cynomolgus monkey C_{H1} sequence. The sequences are numbered according to the system described by Kabat et al. (19). A dash indicates identity to the rhesus monkey C_{H1} sequence, and a dot indicates a gap introduced to maximize alignment.

AGA CAC GGA GAG-3′). Twenty clones were obtained that included three unique sequences, as shown by the amino acid alignment of the constant regions of three representative clones in Fig. 1. Rhesus IgG1 clones similar to a cDNA clone isolated from cynomolgus monkey (*Macaca fascicularis*) (28) predominated (15 out of 20) and clearly were the simian equivalent of the human IgG1 isotype. The majority of variability between the unique clones was observed within the hinge region bridging the first and second constant domains. The two other clones (γ18 and γ20) were not related to any particular human gamma chain, although the hinge region of γ18 showed significant similarity to a human IgG pseudogene (19).

One of the rhesus IgG1 clones was used to generate a macaque heavy chain expression vector. The rhesus clone (a *Xho*I-*Bsa*BI fragment) was substituted for the human homologue in the human heavy chain eukaryotic expression plasmid pCDHC68b (SmithKline Beecham Pharmaceuticals, King of Prussia, Pa.) (42). This created the macaque heavy chain expression vector pMmHCγ1, in which the gene was under the control of the human cytomegalovirus (CMV) promoter and included the amplifiable marker dihydrofolate reductase (dhFr) for selection purposes. The variable domain of the heavy chain portion of Fab 201 was then inserted into pMmHCγ1 using conserved *Xho*I and *Kas*I sites to generate pMmHCγ1-201. To generate a light chain expression plasmid, the light chain of Fab 201 was inserted into the human light chain expression vector pCNHLC under the control of the human CMV promoter (SmithKline Beecham Pharmaceuticals) using *Sst*I and *Xba*I sites to create pMmLCκ-201. This light chain vector encoded the neomycin resistance gene for selection in mammalian cells. Both clones contained the simian virus 40 origin of replication to allow expression in COS cells.

Expression and secretion of IgG were confirmed by cotransfection of COS cells with pMmHCγ1-201 and pMmLCκ-201 (data not shown). Stable IgG1-201-expressing cell clones were then established by cotransfection by electroporation of 10 μg each of the linearized heavy (pMmHCγ1-201) and light (pMmLCκ-201) chain plasmids into Chinese hamster ovary (CHO) cells lacking the gene for dihydrofolate reductase. Clones were selected with 300 μg of G418 sulfate (Gibco/BRL, Gaithersburg, Md.) per ml. Individual CHO clones were isolated and screened for antibody expression by enzyme-linked immunosorbent assay (ELISA) for IgG in the culture supernatant using goat anti-human IgG to coat plates and alkaline

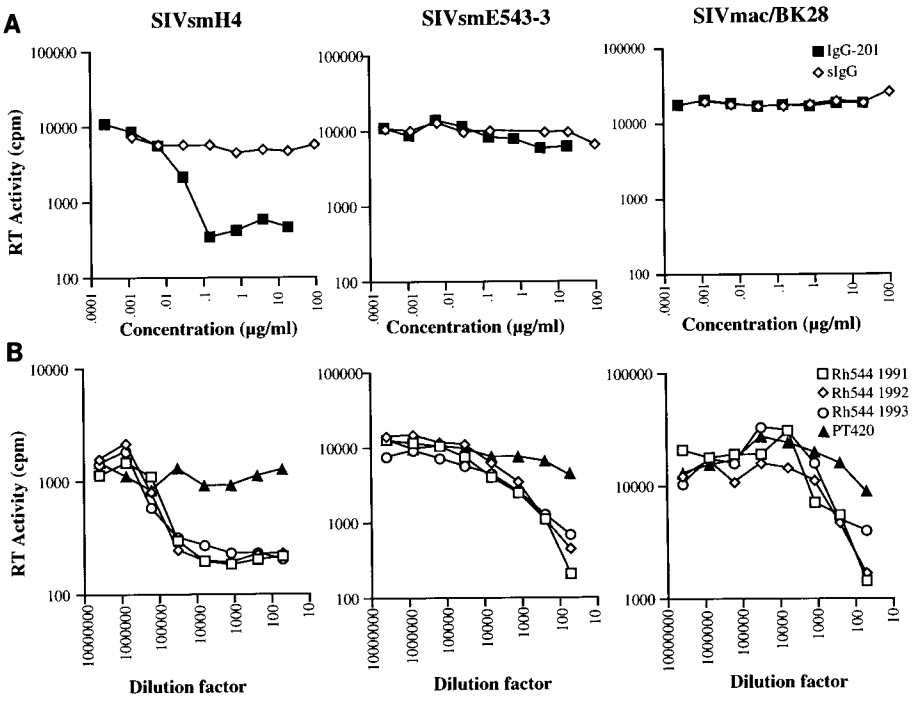

FIG. 2. In vitro neutralization of various molecularly cloned simian immunodeficiency viruses. (A) Neutralization profiles of IgG1-201 and polyclonal rhesus IgG (sIgG) against SIVsmH4 (17), SIVsmE543-3 (16), and SIVmac251/BK28 (23, 26); (B) neutralization profiles of sequential plasma samples from the donor monkey RhE544. Cell-free virus stocks were produced by transfection of the various clones into 293 cells by the calcium phosphate method (CellPhect kit; Pharmacia). Each stock was titrated for infectivity on CEMx174 cells. The neutralizing assay used inhibition of RT as an endpoint. Plasma samples were heat inactivated by incubation at 56°C for 30 min, diluted with phosphate-buffered saline, filtered, and stored at −20°C until use. Fivefold dilution series of purified antibody or plasma were prepared in triplicate, mixed with 100 $TCID_{50}$ of virus in a 96-well plate and incubated for 1 h at 37°C. Exponentially growing CEMx174 cells (2×10^4) were added to each well. Cultures were fed at day 3 with a medium change of half of the culture supernatant, and on day 5, the supernatants were harvested and the RT transcriptase activity was determined.

phosphatase-conjugated goat anti-human IgG Fc antibody (Pierce, Rockford, Ill.) for detection. Macaque polyclonal rhesus IgG was used to establish a standard curve for this assay. Those CHO clones that expressed the highest amount of antibody were selected for gene amplification with methotrexate. A stepwise increase in the concentration of methotrexate (5 μM, 10 μM, and 50 μM) resulted in 100-fold amplification of secretion (a maximum concentration of approximately 8 μg/ml). Analysis of this protein G-purified IgG1-201 by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) revealed the expected light and heavy chain proteins (data not shown).

To evaluate the functional activity of the recombinant antibody, the ability of IgG1-201 to neutralize various SIV isolates was assessed using an assay based upon reduction in reverse transcriptase (RT) activity in culture supernatants. To ensure viral homogeneity we utilized molecularly cloned viruses as targets for neutralization. Cell-free virus stocks were produced by transfection of the various clones into 293 cells by the calcium phosphate method (Pharmacia, Piscataway, N.J.) and 100 50% tissue culture infectious doses ($TCID_{50}$) of each virus stock were used for neutralization assays. As shown in Fig. 2, IgG1-201 efficiently neutralized SIVsmH4 (90% reduction in RT activity) but failed to neutralize the homologous neutralization-resistant strain SIVsmE543-3 (16) or the heterologous, laboratory-adapted strain SIVmac251/BK28 (26). To determine whether this strain-restricted pattern of neutralization was representative of the neutralizing activity in the serum of the donor animal, sequential plasma samples from the donor macaque (RhE544) were analyzed for neutralizing activity using plasma from an uninfected macaque (PT 420) as a negative control for neutralization. As shown in Fig. 2, plasma samples from RhE544 (from 1991 through 1993) efficiently neutralized SIVsmH4 but were much less effective in neutralizing either SIVsmE543-3 or SIVmac251/BK28. Thus, while 90% reduction endpoints were achieved with SIVsmH4, endpoints of only 50% were achieved with SIVsmE543-3 or SIVmac251/BK28.

Our previous studies with Fab 201 suggested that it bound a conformational epitope spanning the V3 to V4 region of the SIV envelope, since it competed with the mouse monoclonal antibodies, KK5 and KK9, that had been mapped to bind in this region of the SIVmac envelope (20, 21). To more closely define the epitope recognized by IgG1-201, the ability of IgG1-201 to compete with soluble CD4 (sCD4) was evaluated. To evaluate interactions between IgG1-201 and recombinant sCD4, an ELISA was developed. Briefly, recombinant sCD4 and a mouse monoclonal antibody (sim.4) specific for human CD4 were obtained from the AIDS Research and Reference Reagent Program. ELISA plates (96-well) were coated overnight with recombinant SIVsm gp130 (a gift from Nancy Haigwood), blocked, and incubated with serial dilutions of sCD4 for 3 h at 37°C. Unbound sCD4 was removed by washing with Tris-borate-saline solution supplemented with 0.05% Tween 20, and fixed concentrations of the gp120-specific antibodies IgG1-201 and KK45 were added for 1 h at 37°C using triplicate samples for each concentration of antibody. As a positive control for sCD4 interaction with SIV gp120, sim.4 binding was detected with an alkaline phosphatase-conjugated mouse IgG-specific antibody (Jackson Immunolabs). Prebinding of increasing amounts of soluble human CD4 to recombinant mo-

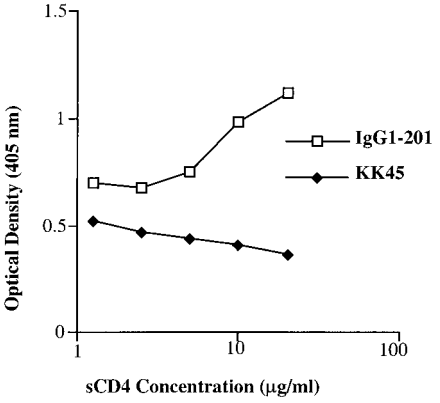

FIG. 3. Enhanced binding of IgG1-201 in the presence of sCD4. Binding of IgG1-201 to SIV gp130 in the presence of increasing concentrations of sCD4. ELISA plates (96-well) were coated with recombinant SIVsmH4 gp120 (a gift from Nancy Haigwood) and incubated overnight. Plates were blocked and incubated with serial dilutions of sCD4 (AIDS Research and Reference Reagent Program, National Institute of Allergy and Infectious Diseases) for 3 h at 37°C. Unbound sCD4 was removed by washing with Tris-borate-saline solution supplemented with 0.05% Tween 20, and fixed concentrations of the gp120-specific antibodies IgG1-201 and KK45 were added for 1 h at 37°C.

nomeric SIVsmH4 gp120 produced in CHO cells actually enhanced the binding of IgG1-201 rather than blocking binding (Fig. 3). As a negative control, the binding of a mouse monoclonal antibody specific for a linear determinant in the V3 loop (KK45) was unaffected by sCD4. These data confirm the CD4 binding domain of gp120 is not a part of the epitope recognized by IgG1-201 but suggests that it may be in close proximity. The enhanced binding defines the IgG1-201 epitope as CD4 induced, similar to the observations for the human HIV-1-specific 17b monoclonal antibody (40, 41).

Since IgG1-201 was effective in neutralizing SIVsmH4 but had no effect on the closely related SIVsmE543-3, we used immunoprecipitation of radiolabeled cell lysates of virus-transfected 293 cells to determine whether IgG1-201 could bind the envelope glycoprotein of both of these viruses. As shown in Fig. 4, IgG1-201 immunoprecipitated envelope glycoproteins of SIVsmH4 but failed to react with the SIVsmE543-3 envelope glycoprotein. Control immunoprecipitations with plasma from an infected animal confirmed that both of these envelope glycoproteins were expressed at detectable levels. These data suggested that the epitope bound by IgG1-201 was either masked or not conserved between the SIVsmH4 and SIVsmE543-3 envelopes. This is an unanticipated finding, since SIVsmH4 is a molecular clone from the SIVsmF236 isolate used to inoculate rhesus E543, the source of SIVsmE543-3, and thus the two clones are closely related (92% identity in envelope).

To more closely confirm the location of the IgG1-201 epitope, a series of chimeric viruses with exchanges of SIVsmH4 fragments into SIVsmE543-3 were generated using conserved restriction sites. Five chimeric viruses containing different segments of the smH4 envelope gene were constructed as detailed in Fig. 4B. These chimeras contained the SIVsmE543-3 genome with substitution of smH4 sequences for the entire envelope (*Bsm*I to *Cla*I; V12345), the V1 to V3 region (*Bsm*I to *Eco*RI; V123), the V4 to V5 region, including the CD4 binding site (*Eco*RI to *Cla*I; V45), the V4 region (*Eco*RI to *Spe*I; V4), or the V5 region (*Spe*I to *Cla*I; V5). Each chimeric envelope was cloned into the 3′-half clone of SIVsmE543-3 using the *Bsm*I and *Cla*I sites. Full-length infectious clones were generated by ligation of the *Csp*45I to *Sal*I fragment of the 3′-half clone with the 5′-half clone of SIVsmE543-3, as detailed previously for other chimeric SIV clones (18). The parental and chimeric virus clones were transiently transfected into 293 cells, and the ability of IgG1-201 to immunoprecipitate their envelope glycoproteins was evaluated.

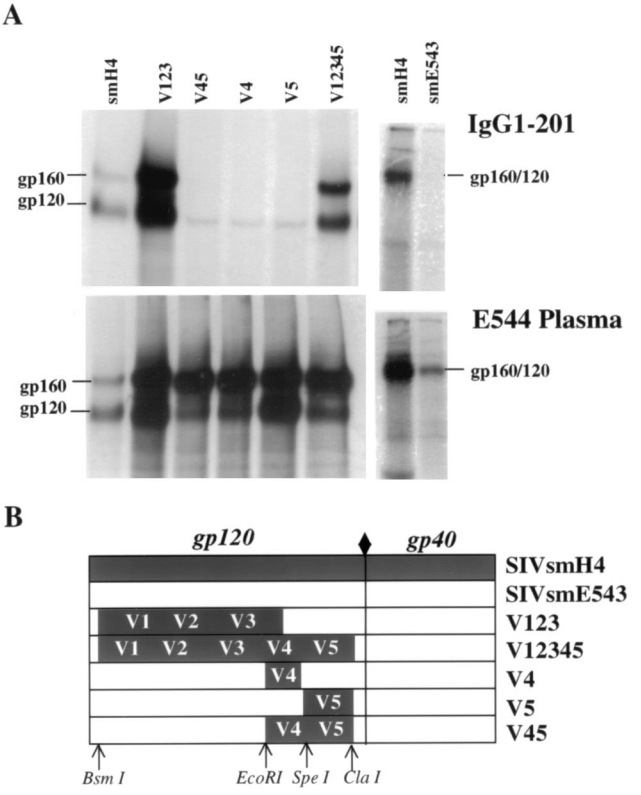

FIG. 4. Radioimmunoprecipitation of envelope proteins from cell lysates of 293 cells transfected with SIVsmH4, SIVsmE543-3, or chimeric envelopes that contained portions of the smH4 envelope (V12345, V123, V45, V4, and V5) as described in the text. 293 cells were transfected with cloned proviral DNA, pulsed with ^{35}S-labeled cysteine and methionine for 24 h, and solubilized in 20 mM Tris buffer, pH 7.5, supplemented with 1% NP-40, 0.5% deoxycholate, and 313 mM NaCl. Radioimmunoprecipitation assays were carried out as previously described (11, 17) using protein A coupled to agarose beads. All samples were analyzed on a SDS–10% PAGE gel under reducing conditions. (A) Immunoprecipitation of the envelope glycoproteins of SIV chimeras from cell lysates of transfected 293 cells with recombinant antibody IgG1-201 (top) and plasma from an SIV-infected animal (bottom). (B) Schematic of the construction of the five envelope chimeras and parental clones used for immunoprecipitation shown in panel A. Sequences from SIVsmH4 are indicated in black and SIVsmE543-3 sequences are indicated in white, with relative locations of variable regions (V1 to V5) and restriction enzyme sites used for construction of chimeras indicated.

As expected, IgG1-201 immunoprecipitated the envelope of the chimera which contained the entire gp120 envelope gene of smH4 (V12345). The only other envelope to be immunoprecipitated by IgG1-201 was from the chimera expressing the V1 to V3 region of SIVsmH4 (V123) (Fig. 4). The absence of binding to the V45 chimera confirmed that the CD4 binding site was not a part of the epitope recognized by IgG1-201. Based upon the proportion of precursor gp160 and gp120 visualized in immunoprecipitations, two of the chimeric envelopes exhibited an envelope-processing defect (V4 and V5). These virus supernatants displayed poor infectivity (data not shown) and were not evaluated further. However, as predicted by the binding assay, the chimeric virus that expressed the V1

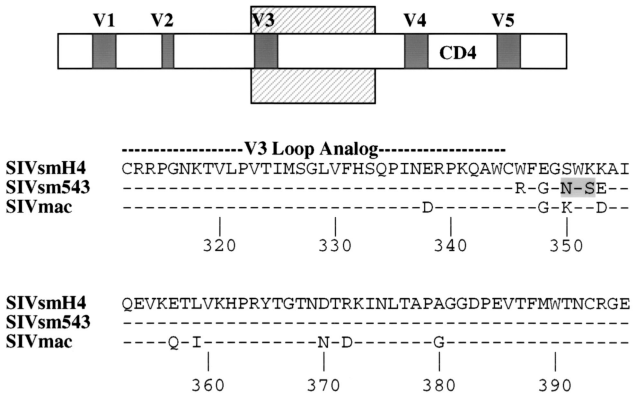

FIG. 5. Alignment of SIVsmH4, SIVsmE543-3 (16), and SIVmac amino acid envelope (23) sequences from the V3 loop analog to the *Eco*RI site, the presumed area of the IgG1-201 epitope binding. Schematic representation of the envelope is shown above, with the region of the alignment designated by a cross-hatched box. The SIVsmH4 sequence is shown on the top, with dashes for SIVsmE543-3 and SIVmac below indicating amino acid identity. The SIVmac sequence is conserved between SIVmac239 and SIVmac251/BK28. The shaded box indicates a unique glycosylation site in the SIVsmE543-3 envelope.

to V3 domains of SIVsmH4 (the neutralization-sensitive virus clone) was efficiently neutralized by IgG1-201 (data not shown).

In a previous study (13), the epitope recognized by Fab 201 was mapped indirectly by competition ELISA with monoclonal antibodies of known specificity. Fab 201 competed with mouse monoclonal antibodies (KK5 and KK9) that react with a conformation-dependent epitope in the V3 to V4 region (7, 21). Previous studies with these mouse monoclonal antibodies have demonstrated that they are highly sensitive to substitutions within the V4 region (25). However, the V123 chimera but not the V4 or the V45 chimera was recognized by IgG1-201, restricting the region of the epitope to the V3 to C3 region. Examination of the amino acid sequence of this portion of the envelope revealed six substitutions in SIVsmE543-3 relative to smH4 (Fig. 5). Two of these substitutions (S-350-N and K-352-S) are responsible for introduction of an N-linked glycosylation site that is unique to SIVsmE543-3. This novel glycosylation site may be responsible for masking the neutralizing epitope in SIVsmE543-3. However, further site-directed mutagenesis will be necessary to confirm this hypothesis.

Considering the immunoprecipitation data, CD4-induced binding, and relative substitutions in the envelope sequences of SIVsmH4 and SIVsmE543-3, we believe that residues immediately C terminal to the V3 loop are important components of the IgG1-201 epitope. The biological characteristics of IgG1-201 resemble those of the HIV-1-specific monoclonal antibodies 17b and 48d that block gp120-chemokine receptor binding and recognize an epitope that is induced by binding of gp120 to CD4 (40, 41). These two human monoclonal antibodies neutralize HIV-1 poorly in the absence of interaction with CD4, suggesting that this epitope is masked prior to conformational changes induced by binding to CD4. It will be critical to determine whether exposure of SIVsmH4 or SIVsmE543-3 to sCD4 would enhance neutralization of these isolates by IgG1-201. The present data suggest similarities in neutralizing epitopes between SIV-infected macaques and HIV-infected humans that confirm the usefulness of this model for addressing the role of neutralizing antibody in mediating vaccine protection.

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